Report of the March 5, 2007 Meeting of the Mold Studies Information Group

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INTRODUCTION

Mold present in indoor environments was nominated to the National Toxicology Program (NTP) for toxicological studies (http://ntp.niehs.nih.gov/go/316). The nomination reflected a broad public concern regarding potential non-infectious, adverse health effects of fungal exposures. Recent natural disasters and accompanying media coverage have heightened awareness of the potential health effects of mold exposure. NTP acknowledges the difficulties inherent in evaluating indoor mold exposures such as determining the appropriate test article (whole fungal organism or mycotoxin) and selecting the appropriate fungal species and strain for evaluation, toxicological endpoints, testing strategies, and route of exposure. The toxicological study of mold exposures is further complicated by the biology of these organisms, because they have multiple life stages and the physical (i.e. spores, hyphae) and chemical (mycotoxins, proteolytic enzymes) agents that they produce may vary with life stage and growing conditions.

In May 2004, an Institute of Medicine (IOM) committee concluded (Institute of Medicine, 2004) that there is sufficient evidence of an association between mold and other factors related to damp conditions and several respiratory ailments including upper respiratory tract symptoms, cough, wheeze, hypersensitivity pneumonitis in susceptible persons, and asthmatic symptoms in sensitized persons. However, the IOM committee concluded that there was inadequate or insufficient evidence to make conclusions for many other health outcomes including rheumatologic and other inflammatory diseases, neurological symptoms, cancer, and reproductive effects. Notably, the IOM report indicated that none of these health outcomes met the definition for the category of “limited or suggestive evidence of no association.” The committee also identified numerous research needs and recommended more research in exposure assessment of mold, standardization of definitions (e.g., “dampness”), and laboratory (toxicology) studies in animals to evaluate the effect of chronic exposures to mycotoxins via inhalation. NIEHS/NTP scientific staff recommended that toxicological studies with the whole fungal organism be considered, since it is likely that any noninfectious adverse effects observed in the animals would be a result of their exposure to multiple, biologically active fungal components and not solely to specific fungal mycotoxins. The NTP Board of Scientific Counselors and NTP Executive Committee (EC) both endorsed NTP studying indoor molds and emphasized a need to develop methods for standardized production of fungal organisms and/or specific components and to evaluate potential toxicants at multiple life stages and under varying growth conditions.

NTP STUDIES

The NTP routinely evaluates the potential toxic effect of environmental exposures in rodent models. The NTP performs appropriate toxicity studies (14-day and/or

90-day) in part to provide dose-setting information for its chronic toxicology/carcinogenicity studies and to address specific deficiencies in the toxicology database for the environmental agent.

The general toxicity screens are typically carried out as contracted studies at several commercial laboratories in the United States. Although designs are flexible, these studies usually involve exposures of rats and mice of both sexes to chemicals or physical agents for periods of 14 to 90 days (Appendix 1). Chronic studies in laboratory rodents remain the primary method by which chemicals or physical agents are identified as having the potential to be hazardous to humans. The NTP long-term toxicology and carcinogenicity studies (bioassays) in rodents generally employ both sexes of rats and mice with three exposure concentrations plus untreated controls in groups of 50 animals for two years.

INFORMATION GROUP MEETING

In March 2007, NTP asked scientists familiar with the challenges in mold toxicology to provide expert input that would assist NTP in the design and conduct of animal toxicity studies on mold (Appendix 2). The scientists met at the NIEHS and were asked to address how NTP might employ exposure scenarios (including test substances, route, duration of exposure) that closely mimic real world human exposure circumstances to reveal the range of biological responses in laboratory animal models and identify potential hazards to human health. The attendees were divided into three working groups to provide input on specific topics: (1) samples and methods of exposure, (2) dosimetry and biomarkers, and (3) toxicological endpoints and health effects. The following three sections summarize the discussions and recommendations of these groups.

Group 1 - Samples and Methods of Exposure

How should NTP make decisions about which mold exposure to test? What would be the features NTP should consider in selecting a relevant exposure to mold? What is the most relevant exposure (e.g., of greatest concern, most widespread, most representative, etc) for NTP to consider studying?

The group felt that the ideal testing scenario would be to study animals in whole-body chambers with real material (dust) from water-damaged[1] building materials, monitor their exposures, and characterize samples from the exposure chambers. This approach would address all aspects of fungal exposures (multiple colonies, glucans, proteases, toxins, fragments, whatever the building materials contribute,

[1] While the information groups frequently focused on mold exposures in water-damaged buildings, the NTP recognizes that mold can be a problem in buildings with damp conditions due to excess humidity that would not be considered water-damaged.

etc), except microbial volatile organic compounds (MVOCs). It would also take into account the effects of growth medium and co-culture scenarios that occur in human exposures (Penttinen et al., 2006; Murtoniemi et al., 2005; Murtoniemi et al., 2002). The use of dust as a test article would miss MVOCs; however, the group placed MVOCs at the bottom of the priority list. The group considered 3 possible exposure routes: intranasal instillation, nose only inhalation (limited length of exposure but defined route), and whole body inhalation (exposure can be longer, but there is oral exposure from grooming). The members noted that typical laboratory animal studies may not be representative of real-life mold exposures in humans, which would likely occur over extended time periods each day, and suggested that it would be useful to have a controlled exposure as is done in an NTP inhalation study. The group indicated that temperature and humidity requirements should be dictated by standard animal housing concerns and not altered for these studies.

The group indicated that there is much overlap in types of molds from damp and water-damaged buildings in different geographical areas (Chew et al., 2006; Rao et al., 2007; Vesper et al., 2006; Vesper et al., 2007a) and that water-damaged buildings can be represented by multiple exposure scenarios. The group suggested that if "real life" exposures were a goal, then it would be best to test the following scenarios:

1) High humidity (damp building with near condensation conditions – air conditioner blowing on wallboard): *A.versicolor, P. chrysogenum, Wallemia, Eurotium*

2) Saturation conditions (complete wetting of floor coverings and wallboard as occurs during flooding): *Chaetomium globosum, Stachybotrys, Ulocladium*

A control exposure could be a mold such as *Alternaria* which people are commonly exposed to on a regular basis, as it is found in house dust and also in the outdoor environment.

What would be appropriate source(s) for relevant test material to study? Should NTP consider using real-life samples? If so, what would be the best approach for representing real-life exposure (e.g., single exposure site, pool from multiple sites)? If representing an exposure scenario is best (e.g., homes in New Orleans following Hurricane Katrina) which one(s) should NTP consider? Should known mycotoxins, MVOCs, or other well characterized fungal materials be run in conjunction with these studies for comparative purposes?

Appropriate sources for these materials would include sampling from a building with many complaints about mold or a building associated with case reports of illness (substantial water damage/continual moisture); alternatively, a wet/then dry scenario might generate more exposure to mold parts (dried out to form dust). The group said the choice of the most appropriate strains to evaluate is

the most difficult question. The strains could be chosen on the basis of the most biologically active using a luciferase-based assay that looks for activity of mycotoxins (Black et al., 2006). Alternatives would be to (1) select from a list of fungal strains determined to be present in wet buildings, (2) use 2 isolated cultures of each fungal species (maybe one isolate of each species collected from New Orleans), or (3) use a fungal isolate collected relatively recently from a moldy building. The group indicated that this last point is very important and that long-term cultured stocks should be avoided.

In addition to testing "real world" material, the recommended approach would be to grow a defined mixture of mold colonies and use them as a comparative exposure scenario. The group suggested starting with 1-2 dozen relevant species (i.e., responsible for mold growth in water-damaged buildings) and select from that list because they are likely responsible for 90% of current exposures. The list should come from studies of colonizing fungal organisms that actually occur in water-damaged buildings and not from air studies. Using only the list of molds found in allergen extracts would miss many relevant species. The group suggested that mycotoxins should be included because they can be well characterized, but noted that individual toxins may not act alone. The mycotoxins of interest for these studies would be those from molds colonizing building materials, not necessarily those known to occur in foods.

Molds should be grown on building materials and the colonies harvested. The group suggested that the building materials used include gypsum board or wallboard, ceiling tiles, and fiberglass duct liner, and that NTP use more than one source for these building materials and pool them for a more realistic exposure scenario. Colonies should be grown for a defined period of time or to a defined density or life stage and then harvested, mixed, and characterized. Exposure concentrations should be based on the mass of dried material used. The group also noted that it would be extremely important to procure building materials free of biocides. NTP should procure enough material at one time for the entire study. Harvesting of the mold could be done dry with a scraper or HEPA vacuum, or wet with glass beads (Schmechel et al. 2003). Dry dust would be easier to handle, but might produce a less controlled exposure and the tendency to agglomerate would need to be taken into account. Keeping dust dry in a dessicator at room temperature (or lower) would likely be most effective at preventing further growth.

Initial characterization of the exposure material should include an evaluation of the known mycotoxins for the species/strains included, glucans, allergens, particle sizes, protease activity, colony-forming units, and the total number of spores. The air samples taken during the study should also be analyzed for these same parameters to look for any variability over time, although most of these measures would not be expected to change. The exposure material should be reevaluated at multiple time points during the study to ensure stability. As part of the characterization, the group suggested that it would be necessary to

be conscious of the potential for spore germination (within animals) following animal exposure to the molds.

What are possible strategies for emulating real-life exposure to mold in a laboratory setting? What types of methods exist that could be used to expose animals to mold in a laboratory setting?

The relevant duration of exposure was considered to be equivalent to two to five years of a human life. It was suggested that this was in the range of about six weeks for a rat. The group felt that a realistic exposure scenario was 24 hrs per day for some number of weeks. The group was aware of laboratories where inhalation chambers exist, but not of facilities that currently conduct studies of this type.

Mold is a complex mixture of multiple organisms at various life-stages. How likely is it that the NTP will be able to identify the causative agent for any observed toxicological effect(s)? Is this important if our goal is to identify the health effects of a "real-life" mold exposure?

The group felt that if studies were started with a complex mixture of several species of mold, some toxicological effect would likely manifest, but it would then be difficult to tease out the active components. If a complex mixture were used in the final design, it could be tested with a parallel track of candidate molecules/toxins or other "purified" materials. Or these could be done as follow on studies after the chronic study, using more specific routes of exposure such as intranasal or intratracheal. The group felt that if health effects attributable to these exposures were identified, they could be followed up on to develop exposure standards and estimate risk.

How might a study such as the NTP proposes be informative for human health?

The proposed exposure scenarios are close to "real-life;" therefore, any effects identified would be relevant to public health. Well-controlled studies with a well-defined and widely accepted design would be useful, but how they would be applicable to establishing appropriate regulatory controls should be considered in the experimental design.

Group 2 - Dosimetry and Biomarkers

What biomarkers of exposure (i.e. antibodies to mold proteins, etc.) should NTP evaluate in rodent models? Are there specific markers depending upon the types of organisms that should be included in the mold exposure? What information about the mold sample would NTP need to identify the specific set?

Hemolysins may be markers of ongoing fungal exposure, but serum levels of these proteins appear to peak early following exposure in rodent species. Stachylysin was not detectable in patients' serum following exposure to *Stachybotrys*, but this may be due to assay difficulties or the gap between exposure and serum sampling.

Tricothecenes can be measured using polyclonal antibodies, but in goat serum, they appear to peak early and are undetectable after 24 hr regardless of dose. Non-specific carrier proteins in the blood need to be considered for detection. Tricothecenes are also detectable in human serum and indicate chronic low-dose ongoing exposures (Brasel et al., 2004).

Hemolysins appear to be species-specific, so there would be a need to test for multiple organisms. Hemolytic activity and/or inhibition of protein synthesis could also be measured, but direct measurement of hemolysins and tricothecenes provides more specific information. Other mycotoxins and metabolites may have potential utility as biomarkers, but information on kinetics and distribution is lacking for most of them.

There was considerable discussion on how to get accurate measurements of dose. Is there a cumulative effect over time or is a single high-dose exposure sufficient to cause health effects? An important study would be to have animals inhale mycotoxins and measure tissue distribution and health effects in an animal model. Need to provide information on the impact of route of exposure on tissue distribution. Dr. Straus described an assay that will measure tricothecenes in serum, and this assay reacts to some extent with all tricothecenes. The assay can detect serum levels of a number of mycotoxins (Roridin A, E, H and L-2, Satratoxin G and H, Verrucarin A and J, Verrucarol and Isosatratoxin F) with a reported limit of detection of 0.14 ppb) (EnviroLogix, 2007; Brasel et al., 2004).

MVOCs are expected to be measurable in blood or urine but are not currently thought to contribute significantly to chronic disease. They are produced by primary metabolism (growth phase) and are not necessarily species-specific.

Non-covalent protein binding, protein and nucleic acid adducts, and glucans were also mentioned as potential biomarkers, but the group had no specific recommendations on their use.

IgE is a reproducible biomarker of fungal exposure but cross-reactivity should be considered. Specific antibodies would be needed for different species/strains. The group indicated that there is a general lack of availability of reagents and analytical methods for these types of studies and a significant amount of methods development will be needed.

Individual organisms suggested for testing include: *Stachybotrys chartarum*, *Chaetomium globosum*, *Aspergillus versicolor*, *Penicillium chrysogenum*.

Stachybotrys chartarum should be the model organism because more is known about it than other fungi.

Given the adverse effects reported in published animal studies are there specific biomarkers of effect that should be assessed?

The group suggested that there be a focus on immunological endpoints and inflammatory responses in the lung.

How would NTP quantify internal dose of the mold exposure? Is it feasible or informative to evaluate tissue distribution following mold exposure?

While it may be feasible to look at tissue distribution of certain fungal components, the group cautioned that spores, fragments, and mycotoxins would likely distribute to tissues differently following an inhalation exposure. Semi-quantitative PCR methods have been used for environmental samples and could be adapted for evaluation of tissue burden if fungal genetic material is distributed to the lung and other tissues following inhalation exposure.

Mold is a complex mixture of multiple organisms at various life-stages. How likely is it that the NTP will be able to identify the causative agent for any observed toxicological effect(s)? Is this important if our goal is to identify the health effects of a "real-life" mold exposure?

This group recommended beginning with a single fungal species and conducting exposures with the whole organism. Depending on the health effects, the specific causative agents could be narrowed down for which reagents and assays might need to be developed.

How might a study such as the NTP proposes be informative for human health?

Rodents may be appropriate for toxicology studies on the effects of molds, but may not to be the model of choice for allergy/asthma. Animal studies may be of limited use for predicting human health effects of exposure to mold.

Group 3 – Toxicological Endpoints and Health Effects

NTP routinely conducts its toxicology studies in rodents (rats and mice). Are these appropriate models for the proposed mold studies?

Material to be tested – Initial discussions focused on what should be tested in the study. The group suggested that there is a need to do single organism studies in order for the health effects data to be meaningful. This is not only important for clarity and reproducibility of data, but may be important from the perspective of inherent toxicity of the organism, as different toxins and quantities of toxins are

produced when grown in isolation versus with other organisms. When considering single organism studies, the group considered *Stachybotrys* important because of its prevalence in indoor air, on water-damaged building surfaces and the attention that it has received in the lay press. *Stachybotrys* may occur in as many as 34% of indoor dust samples (Vesper et al., 2007b). It was suggested that *Stachybotrys* may be present normally in building materials and become activated in damp or humid environments (Govert and Levetin, 2003). *Stachybotrys* often competes poorly in mixed culture in vitro and thereby can be missed when looking for it under these conditions. The group indicated that the best scenario would be to look at several individual organisms as well as cohorts of organisms and suggested that 3-5 of the most common organisms found in "normal" homes versus water-damaged homes should be targeted to address questions of dose and levels of exposure. The study endpoints would differ depending on the organism or exposure scenario selected and pilot studies should be conducted to determine appropriate doses of the material to be tested.

Animal Species – Rodents could be appropriate models, but the use of multiple mouse strains might be considered to address host susceptibility. There are some limitations with inhalation exposures in terms of differences in architecture of rodent versus human nasal passages. Rats are ground breathers and may be more tolerant of molds because of the way their respiratory systems have evolved. Humans would likely be more sensitive as upright breathers. It was suggested that most laboratory rodents have positive antibody responses for *Alternaria* (Dr. Charles Barnes, unpublished data). One group member also indicated that her laboratory has found significant levels of molds in the bedding and food of laboratory rodents (Dr. Ginger Chew, unpublished data) and that this exposure may complicate the observation of health effects from additional mold exposures.

The IOM report specifically mentions data gaps in neurotoxicity, autoimmunity, and several other areas. What would be the most appropriate animal models to evaluate these health effects? NTP routinely includes clinical chemistry, clinical pathology, and histology endpoints in its 14- and 90-day toxicology studies. Should NTP consider evaluation of toxicological endpoints other than those routinely evaluated in its studies? If so, what endpoints should be included?

The group suggested that much information could be obtained from standard 14- and 90-day toxicology studies with some additional endpoints and special studies as described below. The group also recommended that in utero exposures be considered, as the developing organism may be more sensitive to the effects of environmental toxins.

Neurotoxicity endpoints – Olfactory endpoints should be added to the standard NTP testing paradigm. There is clear evidence in the literature that there are at least transient effects on specific olfactory parameters including olfactory sensory neuron death (Islam et al 2006). Neurotoxicity can initially be addressed with a

functional observation battery and standard toxicity studies. If effects are observed, there should be additional special studies.

Autoimmunity endpoints – Serum and tissue samples from standard rodent studies should be investigated for markers of inflammation. Serum evaluation should include investigation of antibody subclasses rather than total immunoglobulins. Tissue samples should be archived for research questions such as the presence of antibodies to specific fungal products. Histological and clinical pathology evaluation from a 90-day study might suggest potential areas for further investigation through the use of specific autoimmune models. If further investigation is suggested from the subchronic studies, the group recommended investigating strains with Th1, Th2, and hybrid immune phenotypes since immunologic responses may differ depending on the genetic background.

Gastrointestinal and cardiovascular endpoints – In several studies of mold exposures, gastrointestinal effects have been reported (Miller et al., 2003). The group recommended evaluating gastric distress through measurement of loose stools, body weight changes, feeding behavior, and by histopathology. Because weight loss may be transient, the group suggested that body weight and feed intake should be measured often. Cardiovascular effects have also been reported in human studies and may relate to levels of fine particulate matter (Miller et al., 2003; Schwartz 1999). Degradation of building materials by fungi could potentially increase the amount of airborne particulates inhaled along with mold exposures. Cardiovascular endpoints for toxicology studies should include histopathology and levels of serum troponin.

Pulmonary endpoints – Pulmonary lavage was considered to be an important endpoint, and it was suggested that lavage fluid be used to measure cytokines and inflammatory mediators. The group also felt that it would be important to measure the lung burden of particulate materials and spores. Measurement of exhaled nitric oxide could also be used as an indicator of pulmonary inflammation and would be indicative of oxidative stress in the lung. The group suggested that this be done in a subset of animals. Immune complex deposition in the lungs and GI tract would also be important indicators of toxicity and could be investigated using immunohistochemistry. Mucin secretion has also been shown to be a good indicator of toxicity following macrocyclic tricothecene exposure (Islam et al., 2006) and could be used as an additional measure of pulmonary toxicity. The group recommended that measures of pulmonary function be conducted at least once weekly in a subset of animals, for comparison to human studies. The group indicated that it would be preferable if this were done using the Buxco system, so that repeated measures could be conducted in the same animals. The group also suggested that provocation challenges with methacholine or sensitization studies with ovalbumin be conducted in a subset of animals to determine exacerbation of airway hyperreactivity and allergic responses.

Other endpoints and design considerations – Protein adducts to Stachylysin were also considered as an endpoint, but the utility of this measure would depend on the test material used, and larger amounts of serum are required for this measurement than are normally available in rodent toxicity studies. The group suggested that measurement of relevant fungal toxins in various tissues would provide information on target organs and potential health effects. Finally, the group indicated that ergosterol is used in agriculture as a measure of mold exposure, but suggested that levels of this compound might be difficult to measure in laboratory animals because of similarities to steroid hormones and cholesterol.

Mold is a complex mixture of multiple organisms at various life-stages. How likely is it that NTP will be able to identify the causative agent for any observed toxicological effect(s)? Is this important if our goal is to identify the health effects of a "real-life" mold exposure?

Individual organisms with the potential to cause health effects can be identified only if single organism exposures are conducted at some point in the study. Exposure materials must be carefully characterized and exposure measures and dosimetrics must take into account spores, fragments, and other potential toxins. Protease and toxin levels should be measured as exposure metrics, since they will provide information on specific materials that lead to adverse health effects. Although the group questioned the amount of toxin exposure in air, fungal toxins have been documented in air samples (Brasel et al., 2005). There is a need to consider viability when characterizing the spores measured in environmental samples. Some group members suggested that whether the numeric estimates of airborne spores are really reliable as exposure measures is questionable. Published studies have demonstrated health effects in rodents following exposure to *Stachybotrys* spores at levels as low as 30 spores per gram of body weight (Fleming et al., 2004). In vitro studies looking at cytotoxicity (cell killing ability) of air from homes with mixtures of mold exposures have frequently focused on spores (Neilsen et al., 2002; Penttinen et al., 2005).

How might a study such as NTP proposes be informative for human health?

The group concluded that using pure cultures would provide the most easily interpretable information for the public and regulatory agencies. Well-characterized short-term toxicity studies and absorption, distribution, metabolism and elimination studies, such as those routinely conducted by the NTP would provide important information not currently in the literature. As these studies are designed, there is a need to be conscious of persistence and reversibility of any observed health effect, and the group suggested that satellite studies might be needed to address this issue. The use of multiple animal strains (e.g. Th1, Th2 and mice with hybrid immune phenotypes) was considered critical to dissect out the issue of susceptibility in a heterogeneous human population. Genome-wide

array differences from multiple mouse strains could be compared using statistical approaches to provide data on host susceptibility.

Plenary Discussion and Path Forward

During the final discussion, many participants indicated that studies with single organisms would be easier to interpret and would be the preferred approach. However, other participants supported the concept that there should be an attempt to simulate exposures that occur in damp or water-damaged buildings using mixed cultures of organisms obtained from moldy buildings and grown on different building materials. Specific concerns raised regarding the latter approach included the ability to get consistent material for exposures, reproducibility and interpretation of the results. Isolates of single organisms found in damp buildings, such as *Stachybotrys chartarum*, and of common molds to which humans are frequently exposed, such as *Alternaria*, could serve as appropriate controls and provide information that would assist in the interpretation of the mixed culture studies.

The organisms should be grown in bulk to a defined growth stage on a variety of building materials that do not contain biocides, and then harvested and dried. A single batch of each mixture or organism to be tested should be used for the entire study. An initial characterization of the dried material should include an evaluation of relevant mycotoxins, glucans, allergens, particle size, protease activity, colony-forming units, spores, and endotoxin levels. Similar measurements of dust samples collected from exposure chambers should be evaluated at multiple time points during the study. Methods development may be required for measurement of some of these endpoints.

The participants supported the use of current NTP 14- and 90-day studies in rodents using whole body inhalation as the preferred route of exposure for the characterized dust. They also suggested using multiple rodent strains and *in utero* exposures to address issues of life stage and host susceptibility. While the group acknowledged that continuous exposure would more closely approximate some “real-life” exposure scenarios, the standard inhalation study design of 6 hours of exposure per day, 5 days per week was considered adequate for these studies. Other routes of exposure might be useful for ancillary mechanistic studies of specific toxins or specific effects. In addition to the measures included routinely in 14- and 90-day toxicity studies (see Appendix 1), the groups recommended evaluating biomarkers of exposure and effect specific for the organisms used for testing. Host antibodies to fungal products, protein adducts, fungal toxin levels, and spore counts in host tissues were all considered to be important biomarkers. The need for information on tissue distribution and growth of organisms in the experimental animals was also considered significant. It was recommended that pulmonary lavage be conducted on all animals to assess altered production of cytokines and inflammatory mediators and that measures of

pulmonary function and airway hyperreactivity be evaluated in subgroups of animals. In addition to routine clinical chemistry and hematology, it was recommended that serum levels of inflammatory cytokines, total immunoglobulins, and Ig isotypes be quantified. The participants also endorsed collecting tissues for gene expression profiling to evaluate potential mechanistic and genetic contributions to any observed effects. As with the exposure measures, some methods development might be required for measurement of some biomarkers of exposure and effect.

The participants indicated that the standard NTP 14- and 90-day toxicology studies would provide important general information on potential health effects in humans. However, additional endpoints to address specific data gaps or to confirm identified targets were suggested. It was recommended that potential neurotoxicity be evaluated using a functional observation battery, olfactory sensing, and cognitive tests. Particular attention should be paid to the cardiovascular, respiratory, gastrointestinal, and immune systems during histological evaluation as these organs have been reported to be potential targets following exposure to molds.

The participants were supportive that these studies would be informative for human health. They suggested that important information would be provided about which organisms may be causative agents for human health effects, the utility of biomarkers other than IgE as measures of exposure and effect, and dose-dependent effects with particular emphasis on respiratory, immune, and neurologic endpoints. In addition, these studies also have the potential to address issues related to gender and the reversibility and persistence of effects.

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Appendix 1

NTP Toxicology Studies

The following information outlines the design and endpoints routinely investigated in 14- and 90-day toxicology studies.

14-Day Toxicity Protocol

The goal is to identify potential target organs and toxicities and assist in setting doses for the 90-day exposure study.

Treatment:

After a 10- to 14-day quarantine period, animals are assigned at random to treatment groups. The study includes 5 treatment groups each administered a different concentration of test article per sex per species plus a control groups. Each group per sex per species contains 5 animals. The animals receive the test article through a designated route of exposure and the control animals receive vehicle alone. For dosed-feed and dosed-water studies, animals are exposed for 14 consecutive days. For inhalation, gavage and dermal studies, animals are exposed for 12 treatment days, not including weekends or holidays with at least two consecutive treatment days before the terminal sacrifice day. Male mice are housed individually and rats and female mice are housed in groups of five animals per cage except for inhalation and dermal exposure studies in which all rats and mice are housed individually.

	Animals	Sexes	Species	Test Groups	Total
Treatment	5	2	2	5	100
Control	5	2	2	1	20
					120

Observations:

Animals are weighed individually on day one on test, after seven days, and at sacrifice. The animals are observed twice daily, at least six hours apart (before 10:00 AM and after 2:00 PM) including holidays and weekends, for moribundity and death. Animals found moribund or showing clinical signs of pain or distress are humanely euthanized. Observations are made twice daily for clinical signs of pharmacologic and toxicologic effects of the chemical. For dosed-feed or dosed-water studies, food consumption/water consumption shall be measured and recorded weekly.

Necropsy and Histopathologic Evaluation:

Liver, thymus, right kidney, right testicle, heart, and lung weights are recorded for all animals surviving until the end of the study.

A complete necropsy is performed on all treated and control animals that either die or are sacrificed and all tissues are saved in formalin. (Necropsy List, see below)

Histopathologic evaluation is done only on those organs/tissues showing gross evidence of treatment-related lesions to a no-effect level plus corresponding tissues are evaluated in control animals. If specific targets are required they shall be read in the control and highest treatment group and the remaining groups to a no-effect level.

Necropsy List

Adrenal glands	Intestine, large (cecum, colon, rectum)	Oral cavity, larynx and pharynx	Spinal cord	Uterus
Brain	Intestine, small (duodenum, jejunum, ileum	Ovaries	Spleen	Vagina
Clitoral glands	Kidneys	Pancreas	Stomach (forestomach and glandular)	Zymbal glands
Esophagus	Liver	Parathyroid glands	Testes, epididymides and vaginal tunics of testes	
Eyes	Lungs and mainstem bronchi	Pituitary gland	Thymus	
Femur	Lymph nodes - mandibular and mesenteric - bronchial and mediastinal (inhalation studies)	Preputial glands	Thyroid gland	
Gallbladder (mouse)	Mammary gland with adjacent skin	Prostate	Tissue masses	
Gross lesions	Muscle, thigh	Salivary glands	Tongue	
Harderian glands	Nerve, sciatic	Seminal vesicles	Trachea	
Heart and aorta	Nasal cavity and nasal turbinates	Skin, site of application (dermal studies	Urinary bladder	

90-Day Toxicity Protocol

In addition to obtaining toxicological data, the purpose of this study is to determine the treatments for each strain and species to be used in the 2-year toxicology/carcinogenesis study.

Treatment:

After a 10- to 14-day quarantine period, animals are assigned at random to treatment groups. The study includes five treatment groups each administered a different concentration of the test article plus a control group. Each group contains 10 animals per sex per species. The animals receive the subject chemical by a designated route of exposure. Controls receive untreated water or feed or vehicle alone in gavage and dermal studies. For dosed-feed and dosed-water studies, animals are exposed for 90 days after which they are sacrificed with no recovery period. For inhalation, gavage and dermal studies animals are exposed five times per week, weekdays only until the day prior to necropsy. Male mice are housed individually and rats and female mice are housed in groups of five animals per cage except for inhalation and dermal exposure studies in which all rats and mice are housed individually.

	Animals	Sexes	Species	Test Groups	Total
Treatment	10	2	2	5	200
Control	10	2	2	1	40
Special "rats" for clinical lab studies	10	2	1	5	100
Special controls for clinical lab studies	10	2	1	1	20
					360

Observations:

Animals are weighed individually on day 1 on test, after 7 days, and at weekly periods thereafter. Animals are observed twice daily, at least 6 hours apart (before 10:00 AM and after 2:00 PM), including holidays and weekends, for moribundity and death. Animals found moribund or showing clinical signs of pain or distress are humanely euthanized. Formal clinical observations are performed and recorded weekly. For dosed-feed or dosed-water studies, food consumption/water consumption is measured and recorded weekly.

Necropsy and Histopathologic Evaluation:

Liver, thymus, right kidney, right testis, heart, and lung weights are recorded from all animals surviving until the end of the study.

A complete necropsy is performed on all treated and control animals that either die or are sacrificed. All tissues required for complete histopathology are trimmed, embedded, sectioned, and stained with hematoxylin and eosin for histopathologic evaluation.

Necropy List

Adrenal glands	Intestine, large (cecum, colon, rectum)	Oral cavity, larynx and pharynx	Spinal cord	Uterus
Brain	Intestine, small (duodenum, jejunum, ileum	Ovaries	Spleen	Vagina
Clitoral glands	Kidneys	Pancreas	Stomach (forestomach and glandular)	Zymbal glands
Esophagus	Liver	Parathyroid glands	Testes, epididymides and vaginal tunics of testes	
Eyes	Lungs and mainstem bronchi	Pituitary gland	Thymus	
Femur	Lymph nodes - mandibular and mesenteric - bronchial and mediastinal (inhalation studies)	Preputial glands	Thyroid gland	
Gallbladder (mouse)	Mammary gland with adjacent skin	Prostate	Tissue masses	
Gross lesions	Muscle, thigh	Salivary glands	Tongue	
Harderian glands	Nerve, sciatic	Seminal vesicles	Trachea	
Heart and aorta	Nasal cavity and nasal turbinates	Skin, site of application (dermal studies	Urinary bladder	

A complete histopathologic evaluation inclusive of treatment-related gross lesions is done on all early death animals regardless of dose group, all control animals, all animals, and all animals in the highest treatment group with at least 60% survivors at the time of sacrifice plus all animals in higher treatment groups. Treatment-related lesions (target organs) are identified and these organs plus gross lesions are examined to a no-effect level.

Histopathology List

Adrenal glands	Larynx (inhalation studies)	Seminal vesicle
Brain (3 sections including frontal cortex and basal ganglia, parietal cortex and thalamus, and cerebellum and pons)	Liver (2 sections including left lateral lobe and median lobe)	Skin, site of application (dermal studies)
Clitoral glands	Lungs and mainstem bronchi	Spinal cord and sciatic nerve (if neurologic signs were present)
Esophagus	Lymph nodes - mandibular and mesenteric - bronchial & mediastinal (inhalation studies)	Spleen
Eyes	Mammary gland with adjacent skin	Stomach (forestomach and glandular)
Femur, including diaphysis with marrow cavity and epiphysis (femoral condyle with epiphyseal cartilage plate, articular cartilage and articular surface)	Muscle, thigh (only if neuromuscular signs were present)	Testes with epididymides
Gallbladder (mouse)	Nasal cavity and nasal turbinates (3 sections)	Thymus
Gross lesions	Ovaries	Thyroid gland
Harderian glands	Pancreas	Tissue masses
Heart and aorta	Parathyroid glands	Trachea
Intestine, large (cecum, colon, rectum)	Preputial glands	Urinary bladder
Intestine, small (duodenum, jejunum, ileum)	Prostate	Uterus
Kidneys	Salivary glands	

Specific Toxicologic Parameters Evaluated:

Clinical Laboratory Studies

Blood is collected from both sexes of "special study" rats, at days 4 ± 1 and 21 ± 2 and from the core study rats at the end of the study. These are processed for hematology and clinical chemistry determinations. Blood is collected from core study mice at the end of the study for hematology determinations.

Hematology	Clinical Chemistry
Erythrocyte count	Sorbitol dehydrogenase

Mean corpuscular volume	Alkaline Phosphatase
Hemoglobin	Creatine Kinase
Packed cell volume	Creatinine
Mean corpuscular hemoglobin	Total Protein
Mean corpuscular hemoglobin concentration	Albumin
Erythrocyte morphologic assessment	Urea Nitrogen
Leukocyte count	Total Bile Acids
Leukocyte differential	Alanine Aminotransferase
Reticulocyte count	Glucose
Platelet count and morphologic assessment	

Blood for Micronuclei

Blood samples are taken from mice and rats at study termination for micronuclei determinations.

Sperm Morphology and Vaginal Cytology Evaluations (SMVCE)

SMVCE are conducted on core study rats and mice. Mortality, body weight changes and clinical signs of toxicity are used to determine the 3 treatment groups used for the SMVC evaluations.

Additional Evaluations Added on a Selective Basis

Besides the routine evaluations for 14-day and 90-day studies, other evaluations may be added on a selective basis. The addition of these other evaluations is based upon findings in the literature or specific characteristics of the chemical or active agent suggesting a specific organ or metabolic pathway could be a target.

Examples of additional evaluations that NTP has included in its studies:

- Telemetry for detection of heart disease
- Lung lavage fluid analysis for detection of lung inflammation
- Special pathology stains (e.g, for detecting thrombosis, apoptosis, cell cycle, etc.)
- Serum cholesterol/triglyceride analysis for detection of altered lipid metabolism
- Serum hormone analyses (e.g., thyroid, reproductive, glucoregulatory)
- Gene analyses (cardiotoxicity, cancer mechanism characterization)
- Urine chemistry analyses for detection of kidney injury
- Special teratology and reproductive studies
- Immune-function studies
- Tissue p450 enzyme-induction studies

Appendix 2

Mold Studies Information Group

March 5, 2007

Participants

National Institute of Environmental Health Sciences (NIEHS)
Research Triangle Park, North Carolina

Dori Germolec, NIEHS **(Information Group Chair)**

***Samples and Methods of Exposure*:**	
○ **W. Elliott Horner,** Air Quality Sciences **(Chair)**	○ **Michelle Hooth,** NIEHS
○ **Cynthia Smith,** NIEHS **(Rapporteur)**	○ **Laura Kolb,** USEPA
○ **Sam Arbes,** NIEHS	○ **Dan Morgan,** NIEHS
○ **Harriet Burge,** Harvard University	○ **Roger Morse,** Morse Zehnter Associates
○ **Karin Foarde,** Research Triangle Institute	○ **Tiina Reponen,** University of Cincinnati
○ **Brett Green,** NIOSH	○ **Joseph Roycroft,** NIEHS

***Dosimetry and Biomarkers*:**	
○ **Robert Esch,** Greer Laboratories **(Chair)**	○ **Faye Grimsley,** Tulane University
○ **Detlef Schmechel,** NIOSH **(Rapporteur)**	○ **Scott Masten,** NIEHS
○ **Ray Biagini,** NIOSH	○ **Michelle Sever,** NIEHS
○ **Tom Burka,** NIEHS	○ **David Straus,** Texas Tech University
○ **Martin Chapman,** Indoor Biotechnologies	○ **Steve Vesper,** USEPA

***Toxicological Endpoints and Health Effects*:**	
○ **James Pestka,** Michigan State University (**Chair)**	○ **Rajendra Chhabra,** NIEHS
○ **Donald Beezhold,** NIOSH **(Rapporteur)**	○ **Dorr Dearborn,** Case-Western Reserve University
○ **Charles Barnes,** Children's Mercy Hospital	○ **Pat Mastin,** NIEHS
○ **Ginger Chew,** Columbia University	○ **Paivi Salo,** NIEHS
○ **Patricia Chulada,** NIEHS	○ **Marsha Ward,** USEPA

***Observers*:**	
○ **John Bucher,** NIEHS	
○ **Allen Dearry,** NIEHS	
○ **Sam Wilson,** NIEHS	
○ **Mary Wolfe,** NIEHS	

Appendix 3

National Toxicology Program
U.S. Department of Health and Human Services

Mold Studies Information Group Agenda

March 5, 2007

National Institute of Environmental Health Sciences (NIEHS)
Research Triangle Park, North Carolina

8:45 AM **Welcome and Introductions** – Dr. Samuel Wilson, NIEHS
Dr. Allen Dearry, NIEHS

9:00 AM **Nomination Background, Study Concept and Charge to Breakout Sessions** – Dr. Dori Germolec, NIEHS

9:30 AM **Breakout Groups – 101 A, C, Exec Conference Room (refreshments in lobby area)**

- Samples and Methods of Exposure
  Chair, Dr. W. Elliott Horner, Air Quality Sciences
- Dosimetry and Biomarkers
  Chair, Dr. Robert Esch, Greer Laboratories
- Toxicological Endpoints and Health Effects
  Chair, Dr. James Pestka, Michigan State University

12:00 PM **LUNCH**

1:00 PM **Breakout Group Presentations and Discussion**

- Samples and Methods of Exposure
- Dosimetry and Biomarkers
- Toxicological Endpoints and Health Effects

2:30 PM **General Discussion and Path Forward** – Dr. Dori Germolec, NIEHS

3:00 PM **ADJOURN**